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coli as well as from *S. cerevisiae*. Due to the ease of this method, it should be possible to complete 20–25 transformations in 1 hr.

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[36] Optimizing the Biolistic Process for Different Biological Applications

By J. C. SANFORD, F. D. SMITH, and J. A. RUSSELL

Introduction

The biolistic process employs high-velocity microprojectiles to deliver nucleic acids and other substances into intact cells and tissues.[1-4] This process has also been called the microprojectile bombardment method, the gene gun method, the particle acceleration method, and so on. Diverse applications for the biolistic process are rapidly being found for both basic research and genetic engineering.

The biolistic process was originally developed as a means to deliver foreign genes into the nuclear genome of higher plants.[1,2] This is where most efforts have been focused, resulting in successful biolistic transformation of a wide range of tissues in a wide range of plant species.[3-28]

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Transformed plant tissues include cell suspensions, calli, immature embryos, mature embryo parts, meristems, leaf pieces, microspores, and pollen. Transformed species include those that were otherwise impossible or very difficult to transform.[12-14]

The biolistic process has proved to be effective even in very small cell types, and has therefore been useful in transforming diverse microbial species. These include microbial eukaryotes such as yeast and filamentous fungi[29] and algae[30]; prokaryotes such as *Bacillus megaterium*,[31] *Pseudomonas syringae, Agrobacterium tumefaciens, Erwinia amylovora, Erwinia stewartii,* and *Escherichia coli*[32]; and obligate fungal pathogens such as *Uncinula necator*.[33]

The biolistic process first made possible the transformation of organelle

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genomes. Chloroplasts of *Chlamydomonas* can now be routinely transformed,[34,35] yeast and *Chlamydomonas* mitochondria can be biolistically transformed,[36,37] and higher plant chloroplasts can be either transiently[38,39] or stably[40] transformed using the biolistic process.

Most recently, the biolistic process has proved useful in transforming animal cell lines,[41] primary animal cells,[42] and intact animals.[43,44]

While the biolistic process clearly has value, we are still learning how to make the process optimally effective within its diverse fields of application. In the last 2 years we have learned a great deal about how to make the process more effective. This chapter is meant to communicate what we have learned, and to help elucidate for others how they might best go about optimizing the process for their own particular applications. The basic features that must be considered by anyone using the biolistic process are (1) particle accelerator parameters, (2) microprojectile parameters, (3) biological parameters, and (4) experimental design.

Particle Accelerator Parameters

There are several ways of accelerating microscopic particles to supersonic speeds, as is required by the biolistic process. These were outlined by Sanford *et al.*[1] Of these various acceleration methods, the only method that has proved to be of general value thus far is acceleration of microprojectiles on the face of a macroscopic carrier, or "macroprojectile." The macroprojectile is in all cases driven by a gas shock. The gas shock can be derived by use of a chemical explosion (gunpowder),[1] an electric explosion of a water droplet,[11,12] a discharge of compressed air,[15] or by a

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helium shock[45] generated by a rupture-membrane mechanism. The macroprojectile may be any lightweight object that has a front surface that can carry microprojectiles, a back surface that can receive the energy of the gas shock, and sufficient cohesive integrity to withstand the gas shock, sudden acceleration, and violent deacceleration.

While there are now numerous particle accelerator designs in use, we will limit our discussion to the gunpowder-driven PDS-1000 accelerator [previously distributed by Du Pont (Wilmington, DE)] and its helium shock driven retrofit (now distributed by Bio-Rad, Richmond, CA). This focus is due to our familiarity with these acceleration systems, both of which were developed in our laboratory. In addition they are the systems most widely used and are the only ones that are commercially available. Researchers using other accelerator designs can still benefit from the information gained through our experience with these types of devices.

Some people may choose to continue to use the gunpowder-driven apparatus, so we include some discussion of it. However, as the new helium-driven apparatus is dramatically superior,[45] most of our discussion will relate to this improved biolistic system.

Power Source

Gunpowder-driven designs employ standard nail-gun cartridges, as are used in the construction industry. Until now this has been the established biolistic power source, but it has the disadvantages of being somewhat dangerous, uncontrolled, and messy in terms of generating dirty gases and debris within the apparatus.

The new helium-driven apparatus has the advantage that the power source is safer and cleaner, and the power output can be regulated. We have observed that helium is clearly superior to other gases such as compressed air or nitrogen. This was as we expected, because helium is a light gas and expands much faster than other conventional bottled gases, imparting higher velocities to lightweight macroprojectiles. We believe the velocities achieved by the gunpowder and helium power sources are not fundamentally different, based on depth of penetration studies. The gunpowder-driven system seems to have higher velocity in the "epicenter" of a target region, but this is usually associated with a zone of cell death roughly 1 cm in diameter.[19] The helium-driven system does not generally produce a zone of death, and apparently produces higher velocities over a wider target area combined with better dispersal of particles, resulting in a more uniform field of transformation. In all biological systems we

[45] J. C. Sanford, M. J. DeVit, J. A. Russell, F. D. Smith, P. R. Harpending, M. K. Roy, and S. A. Johnston, *Technique* **3**, 3 (1991).

have tested, the helium system has proved dramatically superior to the gunpowder system in effectiveness.[45]

Macroprojectile

The gunpowder system requires a cylindrically shaped, high-density polyethylene macroprojectile, a tight-fitting, relatively long, heavily-armored acceleration barrel, and a special Lexan (Dupont, Wilmington, DE) stopping plate with a very small central aperture.

The helium system employs a 2.54-cm circular Kapton (Dupont) membrane (only 0.06 mm in thickness) as a macroprojectile, which has the following important benefits. Only a short flight distance is needed, as the membrane requires very little time to come up to speed. The less massive membrane can be stopped with a screen, rather than a Lexan disk. Therefore more particles can be delivered without any associated high-velocity "debris," which can be generated from the macroprojectile or stopping plate. In addition, the microprojectiles are accelerated in a dried-down form over a larger surface of the wider macroprojectile, and are subsequently dispersed much more widely and uniformly on impact against the stopping screen.

Vacuum/Residual Gas

Regardless of the apparatus used, the gas overlying the target sample usually must be modified. Most commonly, as much of the overlying air is removed with a vacuum pump as is practical, such that a standard vacuum gauge will read 28–29 in. Hg (about 710–740 mmHg). Higher vacuums are not generally practical because of residual water vapor pressure from the biological sample itself. The strength of the vacuum must be reduced for certain applications. For example, bombardment of mouse skin *in situ* requires a reduced vacuum of approximately 20 in. Hg (about 510 mmHg), or the suction on the tissue can damage cells and reduce expression levels.[44] Likewise, mouse liver tissue will not tolerate a vacuum at any level.[44]

The efficiency of transformation of certain biological targets can be enhanced by flushing the chamber with helium prior to pulling a vacuum, such that the residual gas is helium instead of air. This advantage is quite dramatic in microbial systems, and helium flush can increase bacterial transformation by five- to sixfold,[32] and yeast transformation fourfold (J. C. Sanford, unpublished observation, 1992). However, this advantage is not universal. In tobacco cell suspensions the benefit is small or absent (J. C. Sanford, unpublished observation, 1992).

There are two reasons why the gas overlying the biological sample can

affect biolistic transformtion. The principal reason is that microprojectiles are rapidly deaccelerated as they pass through any gas. By removing most of the overlying gas, the amount of deacceleration can be significantly reduced. Likewise, by using a light gas such as helium, the drag can also be reduced. The smaller the microprojectile, the more dramatic the deacceleration problem. Because bacterial transformation involves a subset of microprojectiles that are extremely small, a helium flush and a strong vacuum become especially important factors affecting biolistic efficiency. A second reason why the gas overlying the sample can be important is that this gas can transmit a potentially damaging shock wave. By reducing the density of the overlying gas, or by using a low molecular weight gas such as helium as an overlay, the severity of such a shock wave can be reduced.

Baffles/Meshes

The acoustic shock/gas blast that is generated during the supersonic acceleration of the macroprojectile can kill cells, especially those cells that are multiply traumatized by also being penetrated by the microprojectiles, and which may also be exposed to a selective medium. Even when such shock is not lethal, it may impair subsequent cell division, growth, and regeneration. Modification of the overlying gas can reduce the intensity of this shock, but only slightly. We have, therefore, attempted to reduce the shock wave further. Very fine meshes placed between the microprojectile launch site and the biological sample have been reported to improve gunpowder-driven biolistic transformation dramatically.[14] We have found that such meshes are indeed effective with gunpowder-driven systems, but are less critical with the helium system. We have also tested a single-aperture postlaunch baffle with the gunpowder system, and a prelaunch baffle with the helium system.[19] To evaluate the severity of the shock to the sample, we developed a "shave cream" assay. This assay simply measures the degree to which a layer of shave cream is disrupted by bombardment. We find that the mesh is more effective than a prelaunch baffle, which in turn is more effective in reducing shock than a postlaunch baffle. In addition, a mesh plus a prelaunch baffle is better than either alone. We believe that the benefit of the mesh with the gunpowder system is in reducing shock-generated trauma to cells, in addition to improving disaggregation, as has been proposed previously.[14] While these results show that mechanical shock trauma to target samples can be reduced by meshes and baffles, biological experiments with the helium system show these devices generally have little or no benefit in terms of increasing the number of stable transformants. We conclude that when suitable settings

The factors that affect velocity interact. For example, an increase in power load can compensate for increased gap distance, decreased macroflight distance, or increased microflight distance. Introduction of a baffle-mesh, or reduced vacuum, may need to be offset by shorter microflight distance or higher power load.

Safety

People who choose to build their own biolistic devices should be aware of certain hazards, and should incorporate features into their devices that make them inherently safer. All devices that have electrical components should include a ground fault interrupter mechanism, because users typically are working, often with wet hands, in a metallic, grounded environment (a laminar flow hood)! The hazards associated with gunpowder charges include premature firing due to heat or impact, exploding acceleration barrels due to barrel blockage, and ejection of high-velocity macroprojectile, cartridge, or other debris from a system that has not been fully sealed before firing. High-pressure helium can be hazardous at the tank source, or from rupture of fittings or tubing. Firing of the rupture membrane of a helium system without the benefit of enclosure or vacuum is extremely loud. Such premature firing could conceivably generate small pieces of high-velocity membrane material, or cause injury to the hearing of the user.

Microprojectile Parameters

Choice of Microprojectiles

Tungsten. Tungsten particles can be obtained in various size ranges from Sylvania (Sylvania Chemicals/Metals, GTE Products Corp., Towanda, PA). These particles are extremely irregular in shape and heterogeneous in size. Although different mean sizes range from 0.5 to 2.0+ μm, their distributions overlap extensively. The advantages of tungsten are that it is very inexpensive, it is available in numerous sizes, each size class represents a broad spectrum of particle diameters, it is easy to coat with DNA, and we have more experience with it than with other particle types. The disadvantages are that it is potentially toxic to certain cell types, it is subject to surface oxidation that can alter DNA binding, it catalytically degrades the DNA bound to it over time, and it is highly heterogeneous in shape and size, which prevents optimization of size for a particular cell type.

Gold. Gold particles are available in a very limited range of sizes from

either Aesar (Johnson Matthey Aesar Group, Seabrook, NH), or from Bio-Rad. Aesar particles tend to be 2 μm or larger, while Bio-Rad particles are smaller. Currently available gold particles are much rounder and more uniform in size than tungsten. A principal advantage of gold particles is their uniformity, which allows for optimization of size relative to a given cell type—assuming one of the few available sizes happens to be optimal. An even more important advantage of gold is that it is biologically inert. Gold is not toxic to any cells we have tested, and is already approved by the Food and Drug Administration (FDA) as a human therapeutic agent. Unlike tungsten, gold does not catalytically attack DNA bound to it. A major disadvantage of gold is that it is relatively expensive. Surprisingly, gold is not stable in sterile aqueous suspensions, and over a period of time it agglomerates irreversibly; therefore it is best to prepare gold particles the day they are to be used. The uniformity of gold is undesirable in the sense that if the correct specific particle size for a certain cell type is not available, then transformation rates may approach zero. Last, while DNA can be bound to gold as well as to tungsten, from our experience gold coating is more subject to variation, associated with slight perturbations of precipitation conditions.

Other Particles. We have tried other high-density particles for use as microprojectiles. In our hands, platinum and iridium particles both yield very poor results. We do not know if the problem is that these particles are suboptimal in terms of their diameter, or if they do not coat well with DNA. We have also tried lower density particles as microprojectiles. Glass needles (~1 mm in diameter, 3–30 mm long) can penetrate cell walls and enter into onion epidermal cells. Likewise, dried cells of *E. coli* and *A. tumefaciens* can be shot into living onion epidermal cells. While these lower density particles have the ability to penetrate cell walls, their reduced momentum dramatically reduces the efficiency (rate) of such penetration.

While lower density particles do not penetrate cells efficiently, they provide intriguing possible advantages. For example, dried cells such as *E. coli*, *A. tumefaciens*, and yeast make ideal biological capsules, which should be capable of delivering plasmids or minichromosomes in a naturally encapsulated form. Such encapsulation could completely eliminate problems with particle size heterogeneity, irregular DNA coating, particle agglomeration, and shearing or abrasion of DNA. Experiments indicate that dried cells of *E. coli* harboring a plant expression vector can be used to bombard tobacco or maize cell suspension cells, resulting in β-glucuronidase (GUS)-expressing tobacco cells.[46] Likewise, intact λ

[46] J. L. Rasmussen, J. A. Russell, and J. C. Sanford, manuscript in preparation (1993).

phage bearing a common yeast transformation vector in its "stuffer" region, used as projectiles to bombard yeast, yield moderate to good rates of yeast transformants.[47] Ideally, such biological capsules could be modified to increase their density so that they would penetrate the target cells more effectively.

When cells that lack cell walls or other types of outer sheath material are used as targets (such as animal cell cultures), high rates of transformation can be obtained even when using low-density particles such as silica particles (GlassMilk; Bio 101, San Diego, CA) (J. L. Rasmussen and J. C. Sanford, unpublished observations).

Particle Size. The size of the particles chosen for biolistic transformation is generally based on the size of the target cells. As a rule of thumb, particles should be roughly one-tenth the diameter of the cell. However, there are examples where this is not true. For example, for intact mouse epidermal transformation, surprisingly large particles (3.9 μm) are effective, for cells less than 20 μm in diameter. On the other hand, particles as small as 1.0 μm are very effective on primary cell cultures of myotubes (40 $\times$ 100 μm in length). A summary of available particles and their attributes and uses is given in Table I.

Coating Particles

Microprojectile coating is one of the most important sources of variation affecting biolistic efficiency. Apparently each time DNA is precipitated, its pattern of precipitation and aggregation is unique and nonreproducible. The precipitation occurs so rapidly that it is nearly impossible to obtain a uniform reaction mixture—especially because gold or tungsten particles are difficult to keep in suspension. Thus, even when we do our best to hold conditions constant we see important differences in transformation efficiency from one microcentrifuge tube (precipitation event) to another. Furthermore, we still experience fluctuations from day to day and month to month that we cannot explain (transformation rates seem to go down consistently in the summer, perhaps relating to humidity). Hopefully, superior and more reproducible coating procedures will be developed. Until then, users should strive to make the precipitation reaction mixture as homogeneous and reproducible as possible.

Various DNA-coating protocols have been published, and the essential components of these protocols are given in Table II. Of these protocols, we can best describe and critically evaluate the protocol we currently use, which is distinctly superior to our previously published protocols.

[47] J. L. Rasmussen and J. C. Sanford, manuscript in preparation (1993).

TABLE I
DIFFERENT PROJECTILES AND THEIR USES

Projectile	Diameter (μm)	Attributes	Proven applications
M5 tungsten (Sylvania)	0.1–1.0	Size heterogeneity, irregular shapes	Bacteria, yeast, possibly meristems, with high velocities
M10 tungsten (Sylvania)	0.2–1.5	Size heterogeneity, irregular shapes	*Chlamydomonas*, yeast, plant cells, animal cell cultures
1-μm gold (BioRad)	~1	Uniform in size, round	Plant cells, animal cell cultures, yeast, approximately the same as M10
1.7-μm gold (BioRad)	~1.7	Uniform in size, round	Larger plant cells, mouse skin
1- to 3-μm gold (Aesar)	1–3	Fairly uniform in size, round	Larger plant cells, mouse skin
2- to 5-μm gold (Aesar)	2–5	Fairly uniform in size, round	Mouse liver, muscle, spleen, intestine
Dried *Escherichia coli*, bearing plant vectors	~1	Very uniform in size, symmetrical	Large plant cells
λ phage with markers	~0.1	Very uniform in size, polyhedral	Yeast, plant cells
Glass fragments	~1 × 3–30	Heterogeneous, glass crystals vary in length	Large plant cells

1. To begin, 60 mg of particles is weighed out, placed in a microcentrifuge tube, and vortexed vigorously in 1 ml 70 or 100% (v/v) ethanol. Surprisingly, the brand of microcentrifuge tube can be important. Tungsten and apparently DNA can stick to the surfaces of some types of tubes. "Treff" microcentrifuge tubes (Tekmar, Cincinnati, OH) work very well. Twice we have switched to less expensive brands, resulting in a dramatic loss in efficiency that was not diagnosed until many experiments were ruined. At room temperature the particles are then soaked in ethanol for 15 min, pelleted by a 15-min centrifugation (15,000 rpm), decanted, washed three times with sterile distilled water, and brought up to a final volume of 1000 μl in a 50% (v/v) glycerol solution. These particles can be stored at room temperature for 1–2 weeks (prolonged storage can lead to oxidation of the surfaces of the particles). It was previously recommended that particles be extensively sonicated while in ethanol. We no longer feel this is beneficial, and under certain conditions can make particle agglomeration worse rather than better, especially when gold particles are used.

TABLE II
VARIOUS PROTOCOLS FOR COATING DNA ONTO MICROPROJECTILES

Protocol	Particles	DNA	$CaCl_2$ (M)[a]	Spermidine (M)[a]	Notes	Ref.
Original method (per shot)	18 μl–0.18 mg tungsten (0.008 mg)	4 μg (0.077 μg)	7.5 μl (0.25 M)	None	First method (obsolete)	2
An improved method (per shot)	25 μl–1.25 mg tungsten (0.125 mg)	5 μg (0.5 μg)	25 μl (2.5 M)	10 μl (0.1 M)	Our early method	6
Current method (per shot)	25 μl–1.5 mg tungsten (0.5 mg)	2.5 μg (0.8 μg)	25 μl (2.5 M)	10 μl (0.1 M)	Our best current method	19, 29–39, 52
DeKalb method (per shot)	Pellet—2.1 mg tungsten (0.7 mg)	245 μl/25 μg (8.3 μg)	250 μl (2.5 M)	50 μl (0.1 M)	A clear improvement over that described in Ref. 6	14
Agracetus method (per shot)	Powder—3.5 mg gold (0.324 mg)	70 μg (6.5 μg)			Involves drying DNA onto gold using a nitrogen gas stream	11

[a] The volume and molar concentration (M) of the stock solutions used to coat tungsten for three shots.

2. For convenience, sterile aliquots of 2.5 *M* $CaCl_2$ and 0.1 *M* spermidine (free-base) are stored at 4 and −20°, respectively. Surprisingly, after several months the frozen spermidine goes bad in the freezer, which on several occasions has lead to a ruinous loss of transformation efficiency, which went undiagnosed for considerable periods of time. Therefore, frozen spermidine aliquots should be made fresh at least once a month.

3. We prepare DNA at a concentration of 1 μg/μl. It appears that contaminating protein is a principal cause of particle agglomeration during coating, and has limited how much DNA can be used effectively. Ideally, DNA to be used for biolistic experiments should be put through several additional phenol extraction steps to remove all traces of protein. If the DNA is very pure and in abundant supply, the amount used for coating can be increased severalfold over what we otherwise recommend, increasing transformation rates. However, when transforming bacteria with M5 particles (effective particle size, ~0.1 μm), we find DNA concentrations should be *reduced* fourfold to 0.25 μg/μl.[32]

4. There are different views on how large an aliquot of particles should be coated at one time. For a long time, our laboratory only prepared aliquots large enough for three bombardments (see Table II). However, we now more typically use double aliquots (sufficient for six bombardments). Other laboratories seem to prepare enough particles in a single vessel for an entire experiment. It is not clear to us yet if larger aliquots yield a more or a less uniform reaction mixture. We describe our "traditional" small aliquot (three bombardment) procedure, which obviously can be increased for larger scale reactions.

5. We begin by aliquoting 25 μl of the tungsten suspension into microcentrifuge tubes. It is important to vortex *continuously* while removing aliquots of the tungsten suspension, to avoid nonuniform sampling.

6. We then add 2.5 μl of the DNA stock, 25 μl of $CaCl_2$ stock, and 10 μl of the spermidine stock, in that order, while the microcentrifuge tube is continuously being vortexed. Continuous vortexing is important to ensure a uniform reaction mixture.

7. The mixture should be allowed to react and to coat the particles for several minutes during continuous vortexing. The coated particles should then be gently pelleted by pulse centrifugation (early protocols called for hard pelleting, which leads to more agglomeration).

Loading Particles

Once particles have been coated with DNA they should be used as soon as possible. This is particularly true when tungsten particles are used, because the tungsten can degrade the DNA. If a full day of bombardment

is planned, we recommend coating particles as they are needed, two to four times during the day.

For the gunpowder-driven system, 50 μl of supernatant is then removed, leaving enough for three bombardments of 2 μl to be loaded onto each macroprojectile. Effort is made to divide the particles accurately—one-third per aliquot (which is difficult), and to place the aliquot in the exact center of the macroprojectile (which takes practice).

For the helium-driven system, *all* of the supernatant is removed, and the pellet is washed in 70 μl of 70% (v/v) ethanol. A second wash in 100% ethanol is optional. The particles are then gently pelleted and brought up in 24 μl of 100% ethanol. The resulting suspension is mixed by dipping the microcentrifuge tube in an ultrasonic cleaner (Branson 1200), and then aliquoted (6 μl) onto Kapton flying disks, again using care to take equal amounts of particles per aliquot, and to place the aliquot in the exact center of the disk. It is important that the disks have been washed in 70% ethanol before use, and are free from grease, fingerprints, and so on, to ensure uniform coating and drying. The suspension can be spread over an area 1 cm in diameter in the center of the disk, using the pipette tip. It is *crucial* that, immediately after loading, the disk be placed in a desiccator until thoroughly dry (~60 sec) and that it is kept there until immediately before use. Exposure to humidity during or after drying *dramatically* reduces transformation rates, apparently due to hygroscopic clumping and agglomeration.[32] For certain applications, the amount of particles loaded can be substantially increased.

We believe that the coated particles are relatively stable while in ethanol (at least for half a day), but that once dried they are unstable. Therefore, we dry particles onto disks as needed, and use them within 1–2 hr.

Biological Parameters

There are several biological parameters that are important for successful biolistic transformation. First, one must have an appropriate gene construct with a promoter that is strong and that will express in desired target tissue. Second, the target cells must be in a state receptive to transformation. Third, there must be high rates of particle penetration and cell survival and growth after bombardment. A detailed discussion of the biological factors that have been important in optimizing biolistic transformation of various species in our laboratory is given below. Most of this information has come from our experiments with bacteria, yeast, and plant cell suspension cultures.

Vector Constructs

Obviously, it is important that appropriate vectors be utilized in biolistic experiments. The vectors must have appropriate reporter or selective genes with appropriate promoters, and may be either replicating or integrative. The size and form of the transforming DNA should also be considered.

In plants, we routinely employ the β-glucuronidase (GUS) gene[48] as a reporter gene for evaluating transient expression. Other laboratories use luciferase or anthocyanin genes as reporter genes. For determination of stable transformation rates we routinely use the neomycin phosphotransferase (NPTII) gene,[49] which confers resistance to kanamycin. Herbicide resistance genes can also be used as selective markers.[13,14]

For dicot plants we usually use the plasmid pBI426 (obtained from W. Crosby, Plant Biotechnology Institute, Saskatoon, Canada), which has a GUS–NPTII gene fusion,[50] driven by a double 35S cauliflower mosaic virus (CaMV) promoter plus a leader sequence from alfalfa mosaic virus. This plasmid yields 10- to 100-fold more transiently transformed tobacco cells than does pBI121 (Clonetech, Palo Alto, CA), which has a GUS gene driven by a single 35S promoter and the NPTII gene driven by the nopaline synthase promoter.

In monocot species, constructs with the alcohol dehydrogenase promoter and intron[5] or the rice actin promoter[51] yield much higher numbers of transformants than do constructs with weaker promoters such as the CaMV 35S promoter.

In bacteria and yeast, we deliver autonomously replicating vectors routinely. However, autonomous replication is not essential in yeast, because very high rates of biolistic transformation are also achieved with integrative (nonreplicating) vectors.[29] Vectors bearing plant replicons are not expected to be stable, but may reasonably be expected to increase the probability of integrative events. Likewise, vectors bearing transposable elements might greatly increase the efficiency of integration,[52] which is a limiting factor in biolistic plant transformation.

Vector size does not appear to be a limiting factor. *Escherichia coli*

[48] R. A. Jefferson, T. A. Kavanagh, and M. W. Bevan, *EMBO J.* **6**, 3901 (1987).
[49] E. Beck, G. Ludwig, W. A. Awerswald, B. Reiss, and H. Schaller, *Gene* **19**, 324 (1982).
[50] R. S. S. Datla, J. K. Hammerlindl, L. E. Pelcher, G. Selvaraj, and W. L. Crosby, *J. Cell. Biochem. Suppl.* **14E**, 279 (1990).
[51] D. McElroy, W. Zhang, J. Cao, and R. Wu, *Plant Cell* **2**, 163 (1990).
[52] J. Laufs, U. Wirtz, M. Kammann, V. Matzeit, S. Schaefer, J. Schell, A. P. Czernilofsky, B. Baker, and B. Gronenborn, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7752 (1990).

plasmid vectors are effective up to the normal size limits of such vectors (20–30 kb). λ phage vectors (50 kb) can be bound to tungsten particles and can yield high transformation rates.[47] Likewise, intact cells (e.g., *E. coli*) can be delivered as biolistic projectiles,[46] indicating that entire chromosomes or genomes might be delivered by this process.

Genes can be biolistically delivered as RNA[2] or DNA, in circular or linear[35] form, and as single-stranded[35] or double-stranded DNA.

Cell Age/Physiology

In general, the optimum targets for biolistic transformation are healthy cells that are receptive to transformation and that can withstand the stresses of the bombardment process. This generally means that "young," actively dividing cells are the best. However, there are exceptions to this rule and the optimum cell age of each species must be determined empirically.

When equal numbers of cells from early-log, mid- and late-log, and stationary cultures of *B. megaterium* strain 7A17 are transformed, cells from early-log phase are transformed most efficiently.[31] There is no difference in transformation efficiency between cells from midlog, late-log, and stationary cultures of *E. coli* JA221.[32] In the yeast *Saccharomyces cerevisiae*, cells from stationary cultures are most efficiently transformed.[29] For tobacco NT1 cell suspensions,[53] early log-phase cells, 4 days past subculturing, give the highest rates of transformation. Cells 6 days old (midlog phase) and older yield dramatically lower numbers of transformants. For 'Black Mexican Sweet' (BMS) corn cell suspension cultures, the frequency of cell subculturing can also affect transformability. BMS cell suspensions subcultured three times per week give higher numbers of transformants than cultures subcultured only once a week.

Cell Size

Many organisms having different cell sizes and some cell organelles have been successfully transformed with the biolistic process. Successful organelle transformation include chloroplasts of the green alga *Chlamydomonas reinhardtii* (10 μm),[34,35] chloroplasts of tobacco (5 μm),[38–40] and yeast mitochondria.[36] The bacteria *B. megaterium* (1.3 × 4.0 μm),[31] *E. coli* (1.1–1.5 × 2.0–6.0 μm), *E. amylovora* (0.5–1.0 × 1.0–3.0 μm), *A. tumefaciens* (0.6–1.0 × 1.5–3.0 μm), and *P. syringae* pv. *syringae* (0.7–1.2 × 1.5 μm)[32] have all been transformed using M5 tungsten particles. A variety of plant cells of various shapes and sizes (20–100 μm) have

[53] C. Paszty and P. F. Lurquin. *BioTechniques* 5, 716 (1987).

also been transformed. Target cell size is a major consideration in selection of particle size and target distance (see Tables I and III).

Cell Density

Cell density is an important parameter for the transformation of both microbes and plant cell suspension cultures. Generally, a uniform lawn of cells one cell layer thick is optimal. This provides the greatest number of targets, without extraneous cells that can interfere with plating or selection.

In bacterial systems, cells that are grown in liquid culture are prepared for bombardment by centrifugation, resuspension, and then spreading them evenly over the surface of the bombardment medium. The optimum cell number per 10-cm petri plate for *E. coli* JA221 and *B. megaterium* 7A17 is 2×10^9 and 1×10^8, respectively. More transformants per plate were produced when 3×10^9 *E. coli* cells per plate were bombarded rather than 2×10^9, but transformed colonies were too dense to count.

For tobacco cell suspension cultures, we routinely collect 5 ml of suspension, which contains a 0.6-ml settled volume of cells, onto a 7-cm diameter filter paper. However, at this density the number of stable transformants can be difficult to count, and thus the cells are often diluted and replated after bombardment (see Cell Handling, Transfers, Selection below).

Osmoticum

The addition of an osmoticum (i.e., a supplemental agent increasing osmolarity) to the bombardment medium can dramatically increase the rates of transformation. We have found this to be true for all microbial species tested and for all plant cell suspension cultures, although the optimum concentration for each species varies. Elevated osmoticum concentrations may work by protecting the cells from leakage and bursting (lower turgor pressure) and may also improve particle penetration itself.

The optimum osmotic concentration for *B. megaterium* 7A17 is approximately 1.5 *M* (0.75 *M* sorbitol plus 0.75 *M* mannitol). The optimum for *E. coli* JA221 is approximately 0.6 *M* sorbitol. For bacteria, the optimum osmotic concentration is generally slightly below the toxic level for the species. One exception is *E. amylovora*, which can grow at 1.0 *M* sorbitol. This concentration of osmoticum, however, interferes with ampicillin selection for pUC118 transformation. In this case, a concentration of 0.05 *M* still significantly increases transformation efficiency while permitting selection for the ampicillin-resistant transformants. When selecting *E. coli* JA221 (Δ*trpES leu*$^-$ *hsdR*$^-$ rec*A*$^-$) transformants on tryptophan dropout

TABLE III
BIOLISTIC PARAMETERS FOR DIFFERENT APPLICATIONS[a]

Species	Cell stage/ preparation	Cell density	Vacuum (in. Hg)	Helium overlay	Target distance (cm)[b]	Power	Particle	Notes (efficiency/shot)	Ref.
Escherichia coli	Log-stationary	2×10^9	29	+	6	1,000	M5	20,000 colonies	32
Bacillus megaterium	Log	1×10^8	29	nt[c]	6	1,000	M5	~20,000 colonies	31
Yeast (*Saccharomyces cerevisiae*)	Stationary	1×10^8	29	+	12.3	1,500	M10	~10,000 colonies	29
Mildew (*Uncinula necator*)	Early sporulation	Not applicable	27	nt	2.8	900	M5	~0.1 stable	33
NT1	4 days after transfer (early log)	0.4 ml scv[d]	28	No	12.3	1,000	M10 or 1-μm gold	~800 colonies	52
NT1 chloroplasts	4 days after transfer	300 mg	28	nt	9.6[e]	900	M10	~400 beu[f]	39
Wheat embryos (half shoot apical region)	4- to 5-day-old seedlings	10 explants/ plate	28	No	2.8	1,200	M10	~160 beu	—
Bean embryos (apical dome)	6–12 hr imbibed	10 explants/ plate	28	No	6.0	1,200	M10	~120 beu	—
Peach embryonic axis	2 days imbibed	10 axes/plate	28	No	6.4	1,200	M10	~380 beu	—
Peach cotyledon	10 days imbibed	5 explants/ plate	28	No	9.6	900	M10	~720 beu	—
Animal cell cultures (e.g., myotubes)	Confluent	confluent—60-mm plate	15	nt	12.3	800	1-μm gold	5–50 ng[g]	41,42
Intact mouse skin	Depilatory exposure	1 cm^2	15	No	0.2	1,200	2- to 5-μm gold	1–60 ng	44
Intact mouse liver	Surgical exposure	1 cm^2	0	No	0.2	1,200	2- to 5-μm gold	0.2–0.4 ng	44

[a] These parameters have proved successful, but have not necessarily been optimized.
[b] Gap distance is 1.0 cm except where noted. Flying disk flight distance is 1 cm.
[c] nt, Not tested.
[d] scv, Settled cell volume.
[e] With gap distance at 0.5 cm.
[f] beu, Blue expression units.

medium that had been transformed with plasmid pKRS101 (Ap^r, *trpE*), the optimum osmoticum was 0.6 *M* sorbitol. However, the cells grow slowly at 0.6 *M* sorbitol, which is not a problem with tryptophan selection, but can interfere with ampicillin selection because of formation of satellite colonies around the transformed colony. A lower concentration (0.2–0.4 *M*) of osmoticum still produces more transformants per plate than no osmoticum but also reduces the incubation time and formation of satellites as compared to 0.6 *M*.

In tobacco cell suspension cultures, 2- to 10-fold higher rates of both transient and stable transformants are obtained when at least 300–900 mOsm/kg H_2O osmoticum is included in the bombardment medium.[55] In most of our experiments osmoticum has consisted of equal molarities of mannitol and sorbitol. Because commercial sources of mannitol may be contaminated with abscisic acid[54] we have also tested the use of raffinose as an osmoticum. Thus far, we have not seen a substantial benefit of raffinose over mannitol/sorbitol. Additionally, raffinose is more expensive and is difficult to keep in solution at high concentrations.

Another benefit to using high osmotic conditions in plant cell suspension cultures is the reduction of background cell growth. If the starting osmoticum is inhibitory but not lethal, all cell growth is initially inhibited and then as the osmoticum concentration is lowered and the cells are placed on kanamycin medium, only the kanamycin-resistant colonies resume growth. For NT1 tobacco cell nuclear transformation a concentration of 0.2–0.4 *M* (i.e., 400–700 mOsm/kg H_2O) supplemental osmoticum appears to be optimal.

For transient gene expression in tobacco chloroplasts a concentration of 0.55 *M* mannitol plus 0.55 *M* sorbitol is optimal.[39]

Tungsten Toxicity

In some cases tungsten microprojectiles are toxic to the target cells. Tungsten particles added to the medium of tobacco cell suspensions reduce cell growth, even at concentrations 10–20 times lower than that delivered to a bombarded plate. At higher concentrations, tungsten can cause extensive cell death.[19] Tungsten also dramatically acidifies the culture medium. This is not the cause for toxicity in tobacco cell suspensions, but medium acidification could be a significant problem in pH-sensitive cells. Tungsten does not appear to be toxic to all cell types (e.g., *Bacillus*).

When tungsten toxicity is believed to be a problem, the best solution would be to use gold or another inert particle type, if the appropriate

[54] J. A. Russell, M. K. Roy, and J. C. Sanford, *In Vitro Cell. Dev. Biol.* **28P**, 97 (1992).
[55] H. Belefant and F. Fong, *Plant Physiol.* **91**, 1467 (1989).

size for a particular application is available. In tobacco cell suspension cultures, bombardment with 1-μm gold instead of M10 tungsten particles increases the recovery of stable transformants.[19] Where the use of gold is not possible, then reduced concentrations of tungsten in the DNA reaction mixture, or reduced loads on the macroprojectile, may be tried. Also, the tungsten concentration in the cell environment may be reduced by washing the cells/tissues soon after bombardment. For pH-sensitive cells, the bombardment medium can be buffered. Tungsten acidification of the medium of tobacco cell suspension cultures is effectively buffered by 10 m*M* 2-(*N*-morpholino)ethane sulfonic acid (MES).[19]

Cell Handling, Transfers, Selection

Bacteria. We have developed a transfer system for bacteria that allows exposure to a high level of osmoticum during bombardment and then facilitates a gradual decrease in osmoticum and imparts selection for antibiotic resistance.[32] The transfer device is a thin agar medium layer (7 ml), which is pipetted onto a piece of supporting filter paper (8-cm diameter) with an extending tab for handling (paper + agar = "pagar"). Cells are spread onto the pagar, dried, bombarded, and then the pagar is transferred on top of a selective medium (21 ml). The selective medium includes enough antibiotic so that the final concentration following diffusion into the total 28-ml volume is correct. The pagar medium should contain the desired concentration of osmoticum. The osmoticum concentration for bombardment may be high enough to slow or actually prevent growth initially, but when the pagar is layered over the selective medium diffusion gradually and gently reduces the osmotic concentration while the antibiotic is diffusing upward.

In all microbial systems tested, resuspended cells are slowly dried onto the surface of the medium shortly before bombardment. In cases in which bacteria are bombarded while the surface of the medium is still moist, transformation efficiency is reduced. Also, bombarding moist plates results in splattering of cells and medium during bombardment, and may contaminate the surfaces of the gun for other users.

Plant Cell Suspension Cultures. To prepare plant cell suspension cultures for bombardment, the cells are collected onto 7-cm filter papers (#1; Whatman, Clifton, NJ) using a Büchner funnel. The filter papers containing the cells are then placed over pagar supports in 100 × 15 mm petri plates. The pagar supports consist of a filter paper with attached tabs (for handling), covered with 10 ml of growth medium containing the desired osmoticum, and solidified with 0.25% (w/v) Gelrite (Kelco, San Diego, CA). The cells are allowed to equilibrate with the osmoticum for at least

1 hr before bombardment. During bombardment, the vacuum should not be pulled higher than 28 in. Hg (about 710 mmHg), or the pagar supports will sometimes flip out of the plates due to sudden degassing of the Gelrite. The bombarded plates are then placed in plastic boxes and are incubated in a culture room at 24° and with indirect light.

On the day following bombardment, the osmoticum in the culture medium is reduced in two gentle steps. First, the tabs are used to remove from the old petri plate the pagar supports and cells, all of which is transferred to the new petri plates containing 10 ml of Gelrite-solidified growth medium without osmoticum. Eight to 10 hr later, the pagar support and cells are transferred to new petri plates containing 20 ml of Gelrite-solidified growth medium without osmoticum.

Two days after bombardment, either transient GUS assays are performed, or the cells are transferred to selective medium. For transient GUS assays, the filter papers containing the cells are transferred to new petri plates and 1 ml of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexyl ammonium salt (X-Gluc) staining solution[12] is pipetted under the filter paper so as not to disturb the cells. The cells are then incubated at 37° for 24 hr, and the number of GUS-expressing blue cells are counted.

To select for kanamycin-resistant cells, the filter papers containing the cells are transferred to 100 × 15 mm petri plates containing 20 ml of NT1 growth medium with 350 mg/liter kanamycin and 0.25% (w/v) Gelrite. Kanamycin-resistant colonies begin to appear in 4 weeks.

One of the critical factors for colony growth in tobacco NT1 cells is the gaseous environment. Wrapping the plates with Parafilm (American National Can, Greenwich, CT) delays the appearance of colonies and reduces the number of colonies recovered.[55] This is likely due to ethylene accumulation in the plates. As an alternative, wrapping the plates with venting tape (Scotch Brand #394; 3M Corporation, St. Paul, MN) gives better gas exchange yet still helps reduce entry of contaminants. With venting tape, however, desiccation of the plates occurs more rapidly. Thus, the cells must be transferred to fresh medium at least every 2 weeks.

With our optimized NT1 protocol, typically 500 to 1500 kanamycin-resistant colonies can be obtained from 1 bombarded plate. However, it is impossible to count all of these colonies if the cells are left on the original filter paper disks. Thus, the cells must be replated to a lower density. The method we use is to dilute the cells 2 days postbombardment at the time of transfer to kanamycin medium. First, the filter paper containing the cells is cut into four equal parts. Each filter paper section is then placed in a 150 × 15 mm petri dish containing 40 ml of NT1 medium with 350 mg/liter kanamycin and 0.25% (w/v) Gelrite. Subsequently, 3 ml of liquid medium is pipetted onto each plate and the plates are swirled to spread

the cells uniformly. Because it is difficult to subsequently transfer the cells until colonies appear, the plates are wrapped with Parafilm to reduce desiccation.

Experimental Design Parameters

When optimizing the biolistic process, each cell system requires special considerations. However, there are some features inherent in the process that create variation for all experiments. For example, there can be major variation between different tungsten–DNA coating events as measured by particle agglomeration and transformation efficiency. To minimize this problem, our experimental designs typically block DNA-precipitation aliquots (microcentrifuge tubes) across treatments, so that a "good" or "bad" precipitation is not confounded with a treatment effect. In addition, we see significant fluctuations in agglomeration and transformation efficiency between days and over months.

When applying the biolistic process to any species or cell type for the first time, certain basic parameters must be optimized empirically in an efficient and rational manner. In nonmicrobial systems, these parameters are best studied using a rapid series of transient gene expression experiments. In microbial systems, stable transformation experiments can be used for preliminary testing, because these can be scored very soon after bombardment. Because certain parameters naturally interact, it is logical to test such parameters using a fractional factorial design.[56] We use this design to optimize the physical parameters (power load, gap distance and target distance) of the helium gun for a new application. When we test 3 values of each of 3 parameters the fractional factorial design reduces treatment size from 27 to 13. The size of such factorial experiments is limited by how many samples can be bombarded in a single day, because we do not consider contrasts between different days to be valid. There are significant variations from shot to shot, with some shots being "failures," hence at least 3 replicates are needed per treatment (preferably 5 to 10). Using a factorial design, 13 treatments (with 5 replicates each) would require only 65 bombardments, which is a reasonable-sized experiment for a single day. The data from the fractional factorial-type experiments can be analyzed using the response surface analysis procedure of SAS (Statistical Analysis System; SAS Institute, Inc., Cary, NC). This analysis gives the combination of the three parameters that would give either a

[56] O. Kempthorne, "The Design and Analysis of Experiments." Krieger, Malabar, Florida, 1983.

maximum or minimum response (number of transformants). Based on the results of the first experiment, a second fractional factorial experiment would follow, refining the optimum values for each parameter.

The optimum conditions for the biolistic transformation of plants depends on the type of tissue to be bombarded. With intact tissues such as leaves, meristems, and cotyledons, particle penetration is often the most limiting factor and higher particle velocity may be required either by using higher power load, shorter gap distance, or shorter target distance. With cell cultures, however, cell injury is generally more limiting than particle penetration and more gentle treatments are needed. Furthermore, optimal bombardment conditions depend on whether the purpose of the experiment is for transient gene expression only (e.g., for testing promoter strength) or whether stable transformants are desired. The highest transient transformation rates are generally obtained with more violent treatments, which give better particle penetration. However, these treatments may injure cells in such a way that while they can still express the gene, they may have impaired cell division or growth. Therefore, optimal treatments for stable transformation will tend to be gentler than is optimal for transient expression studies.

A general scheme for optimizing biolistic stable plant transformation is suggested below. With cell cultures, generally we screen for transient plant tranformation in our initial experiments to optimize biological parameters and then we screen stable transformation in later experiments. First, a plasmid with a strong promoter and a marker or reporter gene must be identified. We routinely use the GUS gene for transient assay experiments and the NPTII gene for selection of stable transformants. A plasmid such as pUC118 without GUS or NPTII is used as a negative control. In all experimental designs, each microcentrifuge tube used for DNA-coating particles (precipitation event) should be treated as a block, and ideally the cells from different culture flasks should be randomly distributed or blocked among treatments as well.

The size of an experiment that can be performed in 1 day depends on the time required for cell preparation. For example, with cell suspension cultures, 2 experienced people can bombard 100 plates in 1 day. A more comfortable experiment size is 60 plates/day. However, when cell preparation time is lengthy, such as with embryo dissection, the maximum experiment size may be only 20 plates. For the actual bombardment step, typically 15 to 30 plates can be done per hour (depending on the operator and the complexity of the experiment).

In plant systems the state of the tissue and the osmoticum concentration in the bombardment medium are the two most important biological

parameters to begin to optimize. Helium gun parameters that are alread known to be optimum for a similar target should be used for initial experi ments (see Table III).

Experiment 1

Purpose: To optimize cell type or cell stage for bombardment.

1. Prepare five to seven plates of each cell type or cell stage on appro priate medium.
2. Set helium gun parameters at 1000 psi, gap distance at 1.0 cm, targe distance at 12.3 cm, and 1 cm flying disk flight distance.
3. Coat M10 particles with pBI505 plasmid (dicots) or pACGUS (mo nocots).
4. Bombard the plates and incubate under standard growth condition for 2 days.
5. Stain the tissues by covering them with x-Gluc solution. The x-Glu solution consists of 0.5 mg/ml x-Gluc dissolved in dimethyl sulfoxid (DMSO), 10 m*M* ethylenediaminetetraacetic acid, disodium salt (EDTA) 100 m*M* sodium phosphate, 0.5 m*M* potassium ferrocyanide, and 0.1% (v/v) Triton X-100.[12] Incubate at 37° for 24 hr and count the number o blue expression units (blue-stained cell clusters) per bombardment.

Experiment 2

Purpose: To determine if osmoticum in the bombardment mediun increases transient transformation rates. (*Note:* This will be further opti mized in experiment 5).

1. Prepare bombardment medium with equal ratios of mannitol an sorbitol at a combined concentration of 0, 0.25, 0.5, 0.75, and 1.0 *M*.
2. Distribute the type of cells or tissues selected from experiment onto the prepared plates. Use six plates (one unbombarded control) pe treatment. Incubate for at least 1 hr.
3. Bombard and stain using the conditions described in experiment 1

Experiment 3

Purpose: To determine optimal helium pressure and target distance fo transient and stable gene transformation. (*Note:* For tissues with a length preparation time, such as meristems, divide into two experiments, tran sient and stable).

1. Using the best cells or tissues and optimum osmotic treatment (determined in experiments 1 and 2), prepare 33 plates to be used fo

transient assays and 33 plates to be used for stable selection. There will be five replicate plates per treatment plus three negative controls for both transient and stable assays.

2. Coat M10 particles with plasmid DNA containing the GUS and/or NPTII genes.

3. Bombard the plates using the gun set at three helium pressures (1000, 1300, and 1600 psi) and two target distances (5 and 12.3 cm). Incubate the plates for 2 days.

4. Stain the cells for transient assays with x-Gluc, and transfer cells for stable selection to medium with kanamycin. Determine the best treatments based on the number of blue expression units or kanamycin-resistant colonies/plants.

Experiment 4

Purpose: To determine the optimal combination of flying disk flight distance and helium pressure for transient and/or stable gene transfer. (Again, this experiment may be divided into two parts.)

1. Using the best tissue and osmoticum concentration (determined in experiments 1 and 2), prepare 48 plates to be used for transient assays and 48 plates to be used for stable selection. There will be five replicate plates per treatment plus three negative controls for both transient and stable assays.

2. Coat M10 particles with plasmid DNA containing the GUS and/or NPTII genes.

3. Bombard the plates using three pressures (ranging from the best pressure determined in experiment 3, up to 2000 psi), three flying disk flight distances (0, 1, and 2 cm) and the best target distance determined in experiment 3. Incubate the plates for 2 days, stain with x-Gluc or transfer to kanamycin medium, and count the number of blue expression units or kanamycin-resistant colonies/plants obtained.

Experiment 5

Purpose: To optimize the concentration of osmoticum in the bombardment medium for stable transformation.

Experimental design will be the same as in experiment 2 but stable transformation will be evaluated.

Experiment 6

Purpose: To determine the best particle type (tungsten or gold) for stable transformation.

When attempting to biolistically transform a *bacterium*, osmoticum concentration is the most important biological parameter. Selecting the wrong osmoticum concentration for the initial experiments can mean the difference between 0 and 2000 transformants per plate. Begin by using physical gun parameters (power load, 1000 psi; gap distance, 1.0 cm; target distance, 6.0 cm) and biological parameters (growth phase, logarithmic; cell density, 2×10^9 cfu/plate) that are already optimized for *E. coli* JA221.[32] In the first experiment determine the range of osmoticum that produces transformants. When we used this approach to transform *E. amylovora*, *E. stewartii*, *P. syringae* pv. *syringae*, and *A. tumefaciens* we were able to transform cells in our initial experiment and determine a range of osmoticum for successful transformation.[32] Biolistic transformation of bacteria differs from plant transformation in that M5 tungsten particles are used as well as a helium flush of the vacuum chamber.

Prior to the first bombardment experiment, a method for selecting transformants must be chosen. Either direct selection (auxotrophic marker or antibiotic marker) or indirect selection (agar overlay containing antibiotic for antibiotic marker, or pagar for antibiotic markers) can be used. Also, an upper limit of osmotic concentration can be determined that allows growth of the recipient bacterium, narrowing the range of concentrations to be tested.

Bacterial Experiment 1

Purpose: To determine the approximate range of osmoticum necessary for transformation of the bacterium.

1. Prepare selective medium with 0 *M* osmoticum, three treatments of sorbitol, and three treatments of mannitol at concentrations between 0 *M* and a concentration close to the concentration that prevents growth. Use 3 plates and 1 control per treatment, a total of 28 plates.
2. Spread 1×10^9 cells/plate from a logarithmic growth culture and allow the plates to dry slowly before bombardment.
3. Control plates are prepared by mixing the DNA-coated tungsten with bacterial cells, which are then spread on the plate surface. The control plates are exposed to vacuum only and no helium blast.
4. Set the helium gun parameters: 1000 psi; gap distance, 1.0 cm; target distance, 6.0 cm.
5. Coat M5 tungsten particles with plasmid DNA.
6. Bombard plates, incubate at appropriate temperature, count putative transformants, and determine whether sorbitol or mannitol produces more transformants per plate and the approximate optimum concentration range.

7. Transformation can be confirmed by plasmid isolation, restriction digest, and visualization by agarose gel electrophoresis.

Bacterial Experiment 2

Purpose: Optimize biolistic parameters.

Prepare a fractional factorial design experiment to determine the optimum helium gun parameters. Test helium pressure (1000, 1300, and 1600 psi), target distance (6.0, 9.2, and 12.3 cm), and gap distance (low, middle, and high). Use medium that contains the osmoticum concentration determined in experiment 6a to give the greatest number of transformants per plate. Repeat using parameters suggested by the results of experiment 6b.

Using the optimum parameters determined in experiments 6a and 6b, optimize the biological parameters in the following experiments.

Bacterial Experiment 3

Purpose: Optimize the osmoticum concentration.

Bacterial Experiment 4

Purpose: Optimize the culture growth phase (early, middle and late logarithmic, and stationary).

Bacterial Experiment 5

Purpose: Optimize the cell density per plate.

Summary

The biolistic process is still rapidly evolving. We do not anticipate further major improvements in biolistic apparatus. There will probably still be further major improvements in particles, DNA coating, and vectors, as well as significant further advances in understanding of biological determinants of cell penetration and survival. The technology has currently reached the point at which it can be readily and reliably used for a wide range of applications. Given the information presented in this chapter, new applications can be optimized fairly readily.

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Recombinant DNA

Part H

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